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Variation in canopy duration in the perennial biofuel crop *Miscanthus* reveals complex associations with yield

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Abstract

Energy crops can provide a sustainable source of power and fuels, and mitigate the negative effects of CO_2 emissions associated with fossil fuel use. *Miscanthus* is a perennial C_4 energy crop capable of producing large biomass yields whilst requiring low levels of input. *Miscanthus* is largely unimproved and therefore there could be significant opportunities to increase yield. Further increases in yield will improve the economics, energy balance, and carbon mitigation of the crop, as well as reducing land-take. One strategy to increase yield in *Miscanthus* is to maximize the light captured through an extension of canopy duration. In this study, canopy duration was compared among a diverse collection of 244 *Miscanthus* genotypes. Canopy duration was determined by calculating the number of days between canopy establishment and senescence. Yield was positively correlated with canopy duration. Earlier establishment and later senescence were also both separately correlated with higher yield. However, although genotypes with short canopy durations were low yielding, not all genotypes with long canopy durations were high yielding. Differences of yield between genotypes with long canopy durations were associated with variation in stem and leaf traits. Different methodologies to assess canopy duration traits were investigated, including visual assessment, image analysis, light interception, and different trait thresholds. The highest correlation coefficients were associated with later assessments of traits and the use of quantum sensors for canopy establishment. A model for trait optimization to enable yield improvement in *Miscanthus* and other bioenergy crops is discussed.

Key words: Bioenergy, canopy duration, *Miscanthus*, phenotype, trait, yield.

Introduction

The impacts of rising population, increased energy use, and climate change make it imperative that improved energy sources are developed that have a reduced or negative impact on processes contributing to global insecurity (Alexandratos *et al.*, 2006; Beddington, 2009; Lutz and Samir, 2010). Greenhouse gas emissions are acknowledged to be responsible for climate change, and >60% of these emissions are attributed to the use of fossil fuels (oil, coal, and natural gas) for energy (Herzog, 2009). The use of renewable energy is an important route to reduce emissions by displacing fossil fuel consumption. Bioenergy is an important renewable energy resource as it can directly replace petrochemicals and deliver energy as heat, liquid transport fuels, or chemical feedstocks. In addition, bioenergy provides a potential route to the sequestration

of carbon in soil that opens up the possibility of energy production with negative carbon balances (Dondini *et al.*, 2009).

The advantages of perennial energy crops have been highlighted, including the superior energy ratios delivered compared with annual crops (Heaton *et al.*, 2004). *Miscanthus* is a perennial grass that has a number of characteristics that make it a suitable energy crop, including high biomass accumulation (Clifton-Brown *et al.*, 2001), effective C_4 photosynthesis in temperate regions (Naidu *et al.*, 2003), and a low requirement for nitrogen fertilizer (Beale and Long, 1997; Lewandowski *et al.*, 2000). Despite these characteristics, *Miscanthus* is a largely undomesticated crop, suggesting considerable potential for its optimization. The majority of the *Miscanthus* crop at present is derived from a clonally

propagated sterile triploid hybrid, *M.×giganteus* (Greef and Deuter, 1993; Hodkinson and Renvoize, 2001), considered to be a natural hybrid between *M. sinensis* and *M. sacchariflorus*. In a survey of *Miscanthus* trials in North America and Europe, yield in *Miscanthus* averaged 22 Mg ha^{-1} (Heaton *et al.*, 2004), with maximum values in good growing regions of 40 Mg ha^{-1} (Clifton-Brown *et al.*, 2001). A primary target of research into developing *Miscanthus* as a bioenergy crop is to increase intrinsic yield, which would increase profitability and fossil fuel mitigation. However, the approaches used to optimize yield in annual food crops, which concentrate on the grain-filling period, and optimization of harvest index, may not readily transfer to *Miscanthus* where total above-ground biomass must be optimized. For example, in wheat, yield increases over a 50 year period were attributed to the use of superior farm machinery and improved varieties with early anthesis, and dwarfing genes which reduced lodging associated with nitrogen fertilization (Austin, 1999).

To optimize above-ground biomass production, the aim is to establish and maintain an efficient canopy throughout the growing season in *Miscanthus*. In addition, to sustain a high net energy ratio, yield increases attained by increased use of fertilizer are unlikely to be acceptable in an energy crop. As a consequence, if *Miscanthus* yield is to be improved, it is necessary to explore different intrinsic yield traits and trait combinations such as maximizing light capture throughout an extended growing season.

The principles of optimizing canopy photosynthesis were described by Montieth (1977) and discussed by Beale and Long (1995) in relation to biomass accumulation. Equation 1 describes W_h, the yield of dry matter at final harvest (g m^{-2}), as the product of S_t, the integral of solar radiation (MJ m^{-2}); ε_i, the efficiency with which radiation is captured (no units); ε_c, the efficiency with which radiation is converted to biomass (no units); and the ratio of η, the amount partitioned into harvested biomass (no units), to k, the energy content of the biomass (MJ g^{-1}) (Beale and Long, 1995).

$$Wh = St \times \varepsilon i \times \varepsilon c \times \eta/k \qquad (1)$$

Some of these factors vary little or are fixed by site (Monteith, 1978; Roberts *et al.*, 1993) and, therefore, to improve W_h for a given photosynthetic type (C$_3$ or C$_4$), the primary target for manipulation is to maximize ε_i (Beale and Long, 1995). An important component of ε_i is canopy duration between establishment and senescence. Increasing canopy duration will increase the amount of light intercepted over the year. This was directly demonstrated in a comparison between maize and *Miscanthus*, where increased canopy duration and, consequentially, higher light interception led to higher biomass accumulation in *Miscanthus* (Dohleman and Long, 2009). Other approaches to maximize yield through manipulating photosynthesis have been discussed, but most strategies have yet to be achieved in practise (Parry *et al.*, 2011), so must be regarded as a longer term aim.

In the current study, variation in canopy duration of 244 *Miscanthus* genotypes was determined and the association between yield and canopy duration traits explored. Canopy duration was defined as the composite of canopy establishment and senescence traits. Individual trait assessments were studied to determine their relative correlations with yield.

Materials and methods

Genetic resources

A total of 244 *Miscanthus* accessions were collected from a number of sources within Europe during 2004 and 2005. These accessions had been brought into Europe since about 1935 by horticulturalists and botanists. Almost all accessions were wild, and included accessions from the European funded projects: EMI (European *Miscanthus* Improvement), FAIR 3 CT-96–1392, co-ordinated by the University of Hohenheim, Stuttgart (Clifton-Brown *et al.*, 2001); and BIOMIS, co-ordinated by Plant Research International in Wageningen (Atienza *et al.*, 2002). The germplasm collection comprised 199 *M. sinensis* genotypes, 36 *M. sacchariflorus* genotypes, and nine *M.×giganteus* genotypes.

Trial site description

The trial site, located near Aberystwyth on the west coast of the UK mainland, is on a sloping field that is relatively exposed to winds from the south and west. Soil taxonomy classifies the soil as a Dystric cambisol (FAO, 1988). The stone fraction (particles >2 mm) was estimated as being ~50% of the soil mass in the 0–40 cm layer. The trial was designed with plants in a 1.5 m grid with each plant located at the centre of the grid square. A 90 cm circle around each plant position was sprayed with RoundUp® and Atrazine, leaving grass paths between the plants. Rhizome from each of the selected 244 genotypes was split and pieces of uniform size planted into 25 cm diameter pots. In April 2005, one clone from each genotype was planted into each of four replicated randomized blocks arranged perpendicular to the main slope. A control clone (*M. sinensis* 'Goliath') was planted in two positions within each replicate block.

Canopy duration

Canopy duration was calculated in 2007 (third growing season) and 2009 (fifth growing season). In both years, plants produced multiple stems, with light interception in excess of 95% in some genotypes. Canopy duration was determined as a composite trait comprising early season assessment of leaf establishment and late season assessment of leaf senescence. In 2007, visual scores were used to record establishment and senescence of the canopy. In 2009, an array of quantum sensors was used to measure canopy establishment through a quantitative assessment of light interception. Quantum sensor data (see 'Light interception by canopy') were used because in the same year a comparison of different assessment methods of assessing canopy establishment indicated that quantum sensors gave the best correlation between canopy duration and yield.

Phenotyping: early season assessment
Visual assessment of canopy establishment
Canopy establishment was assessed directly above in a circle, of ~1 m diameter, centred on the plant. Plants were scored on a scale of 0–10; 0 was no visible plant growth, 1 was 10% ground cover, and 10 was complete ground cover. Plants were assessed approximately every 2–3 weeks between April and June 2007 and 2009.

Image analysis of canopy establishment
High-throughput phenomics studies have utilized digital images in the glasshouse and controlled environments. The use of digital images to record canopy establishment in the field was tested. Photographs were taken from directly above the plant using a 3.34 megapixel Nikon Coolpix 995 digital camera (Nikon Corporation, Tokyo,

Japan) using autofocus. This camera has a swivelling screen to aid framing of images taken above the plant. Images were analysed using MATLAB (MathWorks, MA, USA) and a bespoke image analysis program using two adjustable thresholds to define *Miscanthus* leaf in RGB colour space, one for red and the other for green/blue. Images were visually checked and thresholds manually adjusted to produce the best approximation of ground cover. Plants were assessed approximately every 2–3 weeks between March and June 2009.

Light interception by canopy

Light interception at the base of the plant was assessed using a Delta-T SunScan (Delta-T Devices Ltd, Cambridge, UK) containing an array of 64 photodiodes which was centred to the middle of the plant. Light detected across the array was compared with an external sensor array, Delta-T BF3 (Delta-T Devices Ltd), giving values for direct, diffuse, and total incident irradiance to give irradiance intercepted by the plant. Plants were assessed approximately every 2–3 weeks between April and July 2009.

Phenotyping: late season assessment

Visual assessment of canopy senescence

Senescence was scored by observation of the visible aerial parts of the plant, and was scored on a scale of 0–10. Zero represented no visible leaf senescence, 1 was ~10% loss of green leaf, and 10 was 100% loss of green leaf. Plants were assessed approximately every 2–3 weeks between September and February 2007/2008 and 2009/2010.

Stem length and canopy height

How well visual senescence scores defined the end of the season in relation to yield was tested. The comparison was made with two methods of elongation growth. Stem length was measured from three randomly chosen stems and from the base of the stem to the uppermost differentiated ligule. Canopy height was defined as the height at which plant leaves intercepted most of the available radiation. This did not usually equate to the tallest stem but to the point of 'inflection' of the majority of the leaves at the top of the plant. The canopy height was measured from the ground to the inflection of the majority of the leaves at the top of the plant. Both determinations were made every 2–3 weeks between April and October 2009.

Data treatment

Data interpolation and curve analysis

Dates of achievement of thresholds were recorded as number of days elapsed from 1 January of the year in which the main growing season occurred. Where a threshold occurred between two assessment dates, linear interpolation was used to estimate the date of the threshold for canopy establishment and canopy senescence. Thresholds for light interception and image analysis data were estimated using the MATLAB PCHIP interpolation function (Mathworks), which gave similar results to the above linear interpolation, but was more suited to the quantitative data produced in 2009.

The dates at which stem length or maximum canopy height achieved a maximum value were calculated from an automated curve-fitting routine which determined the dates at which peak values were recorded. The peak values were the abscissa at the maximum ordinate using Excel (Microsoft Corporation, Redmond, CA, USA) plus extra functions provided by XlXtrFun (Advanced Systems Design and Development, Pennsylvania, PA, USA). Maximum values were determined from splined data using the MaxY function.

Calculation of canopy duration

Canopy duration was calculated according to the example data shown in Fig. 1 using a canopy establishment threshold of 3 and a canopy senescence threshold of 7. Scores for each plant were calculated and averaged across four replicates. These data allow testing of
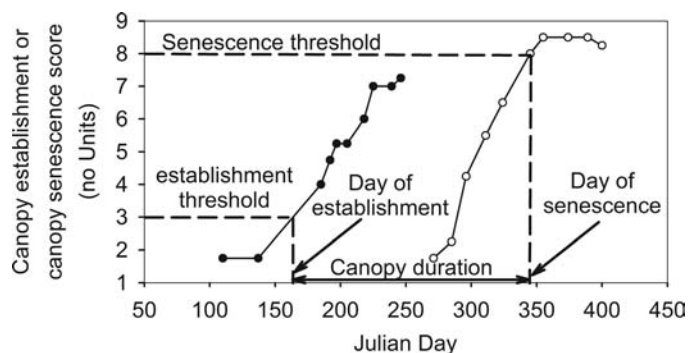

Fig. 1. Illustrative data showing the approach taken to assign canopy durations to *Miscanthus*. Seasonal progression of canopy establishment (filled circles) and canopy senescence (open circles) were used to calculate canopy duration. Canopy duration was the number of days between the day to an establishment score of 3 and the day to a senescence score of 7.

a number of potential thresholds for use in calculating canopy duration. Threshold values were chosen to give long canopy duration, a canopy that was at least 30% green leaf, to include as many available plants as possible in the study, and to produce a high correlation with yield. For example, correlation with yield increased with increasing threshold for canopy establishment and canopy senescence traits, but using higher thresholds reduced the numbers of plants in the analysis because, for example, some stay-green plants do not achieve the higher senescence thresholds. A more quantitative measurement using light interception was used to calculate canopy durations from 2009. A light interception (of threshold of 50% or 30%) was substituted for the establishment score. A threshold of 30% was used to compare with the 30% canopy establishment threshold chosen for the 2007 data.

Group analysis

Data were grouped into genotypic groups to compare yield, the assessed traits, and the calculated canopy duration between the *M. sinensis*, *M. sacchariflorus*, and *Miscanthus* hybrid genotypic groups.

In 2009, different methods of assessing the start and end of canopy duration were compared on a subset of 16 genotypes to encompass a broad range of flowering and senescence phenology. To assess the start of the canopy duration, three protocols were used: a visual assessment of ground cover, image analysis of ground cover from top-down photographs, and a measurement of light interception using an array of quantum sensors. To assess the end of the canopy duration, three protocols were used: a visual assessment of canopy senescence, the point of cessation in canopy height development, and the point of cessation of elongation of individual stems.

Statistical analysis

Statistical analyses were performed using Genstat for Windows [11th edition (2008) VSN International Ltd, Hemel Hempstead, UK]. Genstat was used to determine Bonferroni's test for significance of difference between means. Principal component analysis (PCA) and the coefficient of correlation (R) and associated P-values were calculated using MATLAB (Mathworks). PCAs were calculated on data from 10 d bins of genotypes with high canopy durations from 2007 (canopy durations from 200 d to 209.9 d) and 2009 (canopy durations from 170 d to 179.9 d) that were associated with a large variation in yield. Data analysed included phenological measurements such as seasonal average senescence and day of flag leaf emergence; morphological measurements such as stem number and plant height; and compositional measurements such as lignin (for details

see Allison *et al*., 2011; Jensen *et al*., 2011; Robson *et al*., 2012). Data for PCA were standardized by dividing each trait value by the standard deviation of the associated trait data. For all selected genotypes within the two bins, each individual trait was correlated with yield and the correlation coefficient and its probability calculated.

Results

Individual trait correlations

In 2007, average canopy establishment and average canopy senescence were not well correlated (R=0.1315, P=0.0397) (Fig. 2A). Both were correlated with final harvestable yield (dry weight), with canopy establishment more highly correlated (R= –0.4385, P=0.0000) than canopy senescence (R=0.3659, P=0.0000) (Table 1), although both values were low. In 2009, visual assessment of canopy establishment was replaced by a method based on light interception, and the correlation with senescence was higher than in 2007 (R=0.4694, P=0.0000) (Fig. 2B). Final harvestable yield was correlated better with light interception (R= –0.3497, P=0.0000) than canopy senescence (R=0.23565, P=0.0002) (Table 1).
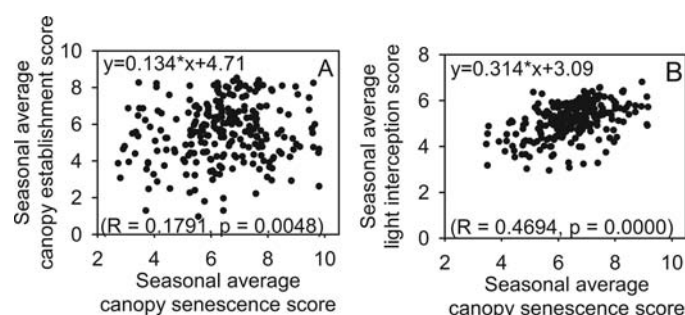

Fig. 2. Associations between average establishment score over ~5 months at the beginning of the growing season and average senescence score over ~6 months at the end of the growing season, illustrating the range of different developmental profiles within the *Miscanthus* population studied.

Trait comparisons between genotypic groups

In 2007, the *Miscanthus* hybrid group had the highest yield and *M. sacchariflorus* the lowest (Table 2). In 2009, the *M. sinensis* and *M. sacchariflorus* genotypic groups were not significantly different for yield and the *Miscanthus* hybrid group was again the highest yielding genotypic group. Yields were higher in 2009 than 2007 in all genotypic groups. In both years, average canopy durations were higher in the *M.* sinensis group, and the other two genotypic groups did not differ significantly (Table 2). Average canopy durations were shorter in 2009 than in 2007, so tests were conducted to determine if this was due to different threshold values of canopy establishment used in calculating canopy duration. When a 30% light interception value was used to match the 30% ground cover assessment used in 2007, average canopy durations remained shorter in 2009 than in 2007 by an average of ~15 d (Table 2).

Trait correlations within genotypic groups

Average canopy durations for genotypes in the *M. sacchariflorus* and especially the *Miscanthus* hybrid genotypic groups were highly correlated with yield (Table 3). Canopy duration in the *M. sinensis* genotypic group was only moderately correlated with yield. Canopy duration correlated highly with canopy senescence, and this correlation was higher in *M. sacchariflorus* and *Miscanthus* hybrids in both years (Table 3).

Trait correlations comparing all genotypes

Average canopy duration was compared with dry matter yield across all individual genotypes (Fig. 3A, C). The relationship between canopy duration and yield data for all genotypes from 2007 was described by a linear curve fit (R=0.6140, P=0.0000) and was similarly described by exponential fit (R=0.6793). The relationship between canopy duration and yield data from 2009 was described by a linear curve fit (R=0.4860, P=0.0000) and an exponential curve fit (R=0.6099). In both years, individual traits correlated with

Table 1. Coefficients of correlation between canopy establishment, canopy senescence, canopy duration, and yield in 2007 and 2009.

	Canopy establishment		Canopy senescence		Canopy duration		Yield		n
	R	P	R	P	R	P	R	P	
2007									
Yield	–0.4385	0.0000	0.3659	0.0000	0.6140	0.0000	–	–	244
Canopy duration	–0.5078	0.0000	0.7522	0.0000	–	–	0.6140	0.0000	244
2009									
Yield	–0.3497	0.0000	0.2356	0.0002	0.4860	0.0000	–	–	244
Canopy duration	–0.1577	0.0132	0.8147	0.0000	–	–	0.4860	0.0000	244
Q4.1	–0.1905	0.2100	0.7395	0.0000	–	–	0.0134	0.9303	45
Q4.2	–0.9339	0.0000	0.0147	0.9570	–	–	0.8524	0.0000	16

Data were analysed for correlation using MATLAB.

The P statistic indicates the probability of correlation occurring by chance, n=number of genotypes. Q4.1 and Q4.2 are two populations from high canopy duration genotypes that separated according to their correlations between canopy duration and yield.

Table 2. Dry matter yield per plant (DM) and canopy duration for three genotypic groups of *Miscanthus* in 2007 and 2009.

Genotypic group	Average DM yield (g plant $^{-1}$) (2007)	Average canopy duration (d) (2007)	Average DM yield (g plant $^{-1}$) (2009)	Average canopy duration (d) (2009)[a1]	Average canopy duration (d) (2009)[a2]
M. sinensis	417.6 b	213.9 b	998 a	177.6 b	201.1 b
M. sacchariflorus	242.9 a	177.9 a	790 a	154.5 a	166.7 a
M. hybrid	590.3 c	194.7 (a)	2029 b	160.3 a	173.5 a

Values in each column followed by different letters are significantly different using Bonferroni's test for significance of difference between means at *P* <0.05.
[a] 2009 canopy durations were calculated using two different threshold values of light interception for establishment: [1]threshold 50%, [2]threshold 30%.

Table 3. Coefficients of correlation between canopy duration and either yield, canopy establishment, or canopy senescence in 2007 and 2009. Data were placed into bins according to genotypic group.

Correlation with canopy duration	Yield		Canopy establishment		Canopy senescence	
	R	*P*	*R*	*P*	*R*	*P*
M. sinensis (2007)	0.6356	0.0000	–0.5768	0.0000	0.6769	0.0000
M. sacchariflorus (2007)	0.6461	0.0005	–0.7947	0.0000	0.7643	0.0000
M. hybrid (2007)	0.8375	0.0000	–0.3258	0.1390	0.9336	0.0000
M. sinensis (2009)	0.5783	0.0000	–0.2794	0.0000	0.7621	0.0000
M. sacchariflorus (2009)	0.7687	0.0000	–0.0048	0.9780	0.9319	0.0000
M. hybrid (2009)	0.9019	0.0000	–0.5991	0.0032	0.8677	0.0000

Data were analysed for correlation using MATLAB; the *P* statistic indicates the probability of correlation occurring by chance.

yield less well than did canopy duration. Canopy establishment correlated better with yield than did canopy senescence. Coefficients of correlation for canopy establishment were negative and for canopy senescence were positive (Table 1). In both years, canopy durations extended over a range in excess of 120 d and both the yield and the variance in yield increased with increasing canopy duration (Fig. 3B, D).

Two populations of high canopy duration genotypes

Graphical analysis comparing canopy duration and canopy establishment data from 2009 in 25% of the genotypes, those with the longest canopy durations (quartile 4), showed two separate populations (Fig. 4). One population, Q4.1, comprised 45 genotypes of which 38 were *M. sinensis*, two were *M. sacchariflorus*, and five were from the *Miscanthus* hybrid genotypic group. A second population, Q4.2, comprised 16 genotypes of which all were *M. sinensis*. The correlation between canopy duration and yield for all genotypes in this quartile was low and not significant (*R*= –0.1764, *P*=0.1736) (data not shown). Data from the 45 genotypes in population Q4.1 showed no correlation between canopy duration and yield (*R*=0.0134, *P*=0.9303). The second population (Q4.2) showed a very strong correlation between canopy duration and yield (*R*=0.8524, *P*=0.0000). Canopy duration was strongly correlated with senescence in population Q4.1 (*R*=0.7395, *P*=0.0000) and weakly correlated with canopy establishment (*R*= –0.1905, *P*=0.2100); and in population

Q4.2 canopy duration was strongly correlated with canopy establishment (*R*= –0.9339, *P*=0.0000) and there was no significant correlation with senescence (*R*=0.0147, *P*=0.9570) (Table 1).

Principal components analysis

At longer canopy durations, greater variation in yield was associated with small variations in canopy duration; for example, in 2009 average canopy durations from 170 d to 180 d were associated with yields ranging from an average of 550 g to 3500 g dry weight per plant. Genotypes within a high canopy duration bin of 10 d from each year of the study were analysed using PCA. The first three components accounted for 72% (2007) and 73% (2009) of the variance. The largest coefficients in the first components in 2007 and 2009 corresponded to maximum canopy height, lignin content, and leaf width; the largest coefficients in the second component in 2007 and 2009 corresponded to maximum canopy height, ploidy, moisture content, and leaf length; the largest coefficients in the third components in 2007 and 2009 corresponded to stature traits such as leaf and stem angle. The average trait value for each genotype within the two 10 d bins from 2007 and 2009 was also individually correlated with yield. The trait that correlated most strongly with yield in both years was canopy height; other stem traits, composition at harvest, and leaf traits also showed significant yield correlations (Fig. 5).
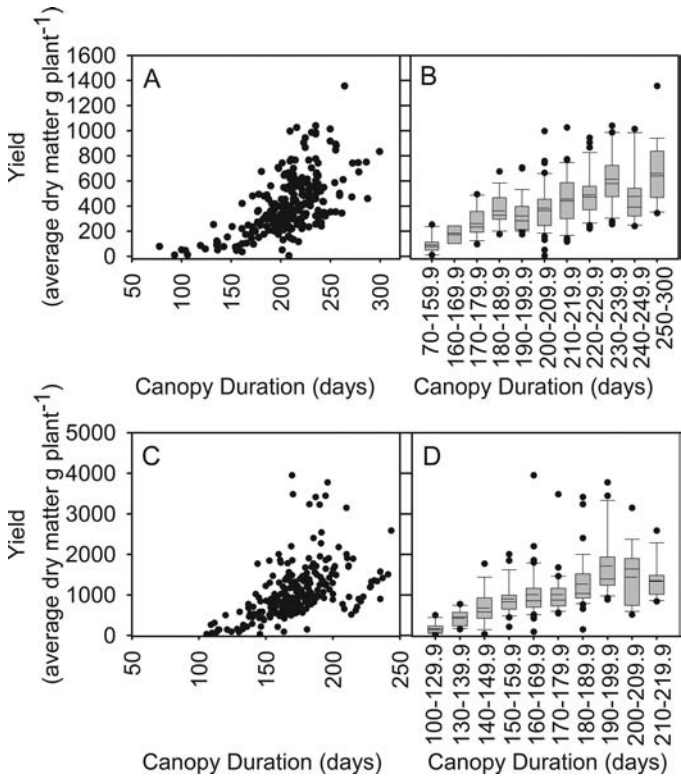
Fig. 3. (A) Correlation between average canopy duration and dry matter yield (g dry matter plant^{-1}) within a diversity trial of *Miscanthus* growing in 2007. (B) Box plot summarizing average dry matter yield per plant for different ranges of canopy duration within a diversity trial of *Miscanthus* growing in 2007. (C) Correlation between average canopy duration and dry matter yield (g dry matter plant^{-1}) within a diversity trial of *Miscanthus* growing in 2009. (D) Box plot summarizing average dry matter yield per plant for different ranges of canopy duration within a diversity trial of *Miscanthus* growing in 2009. In box plots (B and D), outliers are represented as filled circles, the box illustrates upper and lower quartiles, solid lines within the box represent the median and the dashed line the mean. Summary data show in general an increase in yield and variance with increasing canopy duration.
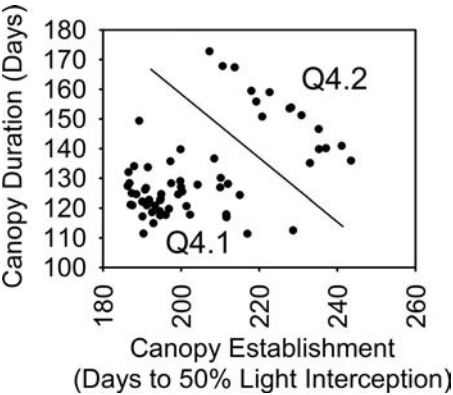

Fig. 4. The correlation between canopy duration and canopy establishment in the quartile of *Miscanthus* genotypes with the highest canopy durations reveals two populations Q4.1 and Q4.2.
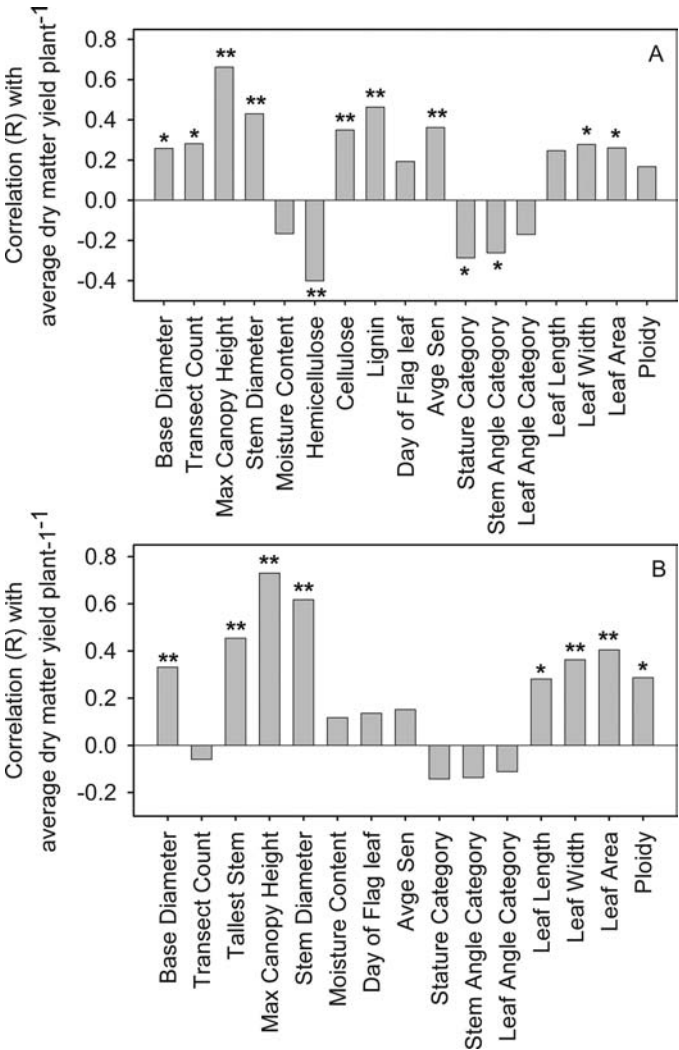

Fig. 5. Individual trait correlations for all available traits from two subpopulations with large yield variances across canopy durations 200–210 [A (2007)] and 170–180 [B (2009)], indicating variation in traits that are associated with yield variance at high canopy durations. *$P > 10\%$; **$P > 5\%$.

Comparing trait assessment methods

The choice of threshold value was investigated using a calculation of R^2 which therefore has no sign and allows direct comparisons of absolute correlation values. The rank order generated by early and late season assessments using different thresholds showed that different traits correlated better when later threshold values were used. For example, the rank order values for genotypes according to the day of maximum canopy height were compared with the rank order values for genotypes according to different senescence thresholds. The rank order correlations for senescence thresholds from 40% to 90% were 0.01, 0.02, 0.09, 0.18, 0.24, and 0.29. This general trend was seen both in early season and late season assessments (data not shown). Higher correlations with yield were calculated when higher thresholds were used to calculate canopy durations (Figs 6, 7).

Correlations with yield were fairly similar when canopy duration was calculated using three different early season
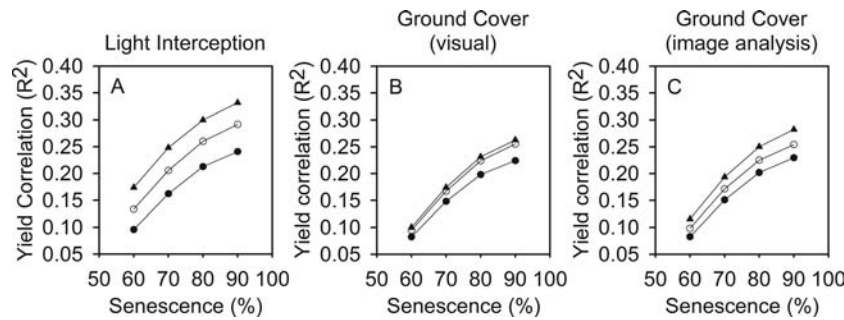
Fig. 6. A comparison of early season canopy assessment methodologies. Different assessment methods were used to define the start of canopy duration and the same assessment (days to 70% senescence) to define the end of canopy duration; the resultant canopy durations were correlated with dry matter yield. For all assessments, later thresholds correlated better with yield than did earlier assessments. All three assessment methods produced roughly similar levels of correlation, with the highest correlations from using quantum sensors (A), the lowest using visual assessment of ground cover (B), and intermediate levels of correlation from image analysis to assess ground cover from digital photographs (C). Canopy durations were calculated using different trait combinations as follows. (A) Filled circles, days between % senescence (on *x*-axis) and 30% light interception; open circles, days between % senescence (on *x*-axis) and 40% light interception; filled triangles, days between % senescence (on *x*-axis) and 50% light interception. (B) Filled circles, days between % senescence (on *x*-axis) and 20% visual ground cover; open circles, days between % senescence (on *x*-axis) and 30% visual ground cover; filled triangles, days between % senescence (on *x*-axis) and 40% visual ground cover. (C) Filled circles, days between % senescence (on *x*-axis) and 20% ground cover from image analysis; open circles, days between % senescence (on *x*-axis) and 30% ground cover from image analysis; filled triangles, days between % senescence (on *x*-axis) and 40% ground cover from image analysis.

assessment methods with the same senescence thresholds from 60% to 90% (Fig. 6). The ranking of correlations with yield for different assessment methods used to calculate canopy duration was light interception > image analysis > visual assessment. The ranges of coefficients calculated using each method

Fig. 7. A comparison of canopy duration calculated using day of 90% canopy senescence assessment (filled circle) and end of season defined as the day to maximum growth measured by either canopy height (filled triangles) or stem elongation (filled inverted triangles). The start of canopy duration was defined by three threshold values for light interception using quantum sensors, and the resulting canopy durations were correlated with dry matter yield per plant. The highest correlation coefficients were achieved using day to maximum stem elongation, the lowest using day to 90% senescence, and intermediate by using day to maximum canopy height. Canopy durations were calculated using different trait combinations as follows: filled circles, days between % light interception (on *x*-axis) and 90% senescence; filled triangles, days between % light interception (on *x*-axis) and day to maximum canopy height; filled inverted triangles, days between % light interception (on *x*-axis) and day to maximum stem elongation.

was 0.10–0.33, 0.08–0.26, and 0.08–0.28 for light interception, visual assessment, and image analysis, respectively.

Canopy durations calculated using senescence and light interception thresholds from 30% to 50% had lower correlations with yield than did canopy durations calculated when senescence was replaced with one of the two elongation assessments to define the end of the growth season (Fig. 7). The ranking of correlations with yield for different assessment methods used to calculate canopy duration was maximum stem elongation > maximum canopy height > canopy senescence. The highest correlations with yield were again obtained from later assessment thresholds in all three methods
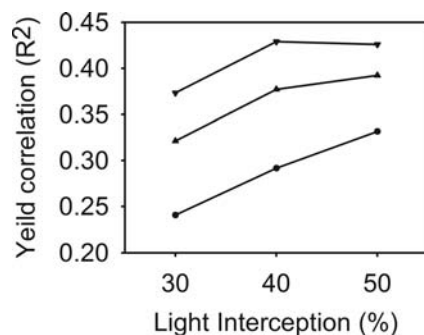
Discussion

How do canopy duration traits correlate across different years?

Canopy durations from 244 *Miscanthus* genotypes growing in a replicated field trial were calculated from assessments of plant phenology over two years, one year in which plants had not yet fully established (2007), and a second in which plants were established (2009) (Tables 1, 3). Both average yield and canopy duration of the population varied between the two years. Canopy establishment and canopy senescence were advanced in 2009 compared with 2007. This was especially significant for senescence and was probably due to increased biomass in more mature canopies promoting higher levels of leaf senescence. The effects of other environmental factors cannot be excluded; however, meteorological conditions were similar in both years (Robson *et al.*, 2012). The range of canopy durations was high and ranged from 78 d to 299 d in 2007, suggesting that this diverse *Miscanthus* collection was suitable to examine the relationship between canopy duration

traits and biomass yield. Genotype averages were the mean across four replicate blocks and represented the genotypic variation in the traits studied.

The low correlation (Fig. 2) between canopy establishment and canopy senescence suggests that it may be possible to select for extremes of both traits to optimize seasonal duration if this was predicted to be of benefit. This appears to be the first example that compares establishment and senescence in a diverse range of genotypes. For a given growth rate, leaf senescence optimizes nitrogen use (Hikosaka, 2005) and therefore it might be predicted that senescence progression would parallel canopy establishment. Variation in traits such as growth rate, stem number, leaf area, and chlorophyll content, and variation in phenological traits such as flowering, will contribute toward reducing the correlation between canopy establishment and canopy senescence in this diverse range of genotypes.

How does canopy duration relate to yield?

The hypothesis that increased canopy duration was associated with increased yield was tested. The relationship between canopy duration and yield was best described by an exponential fit [R=0.6802 (2007) and R=0.6099 (2009)], indicating that yield increased with greater canopy duration for a proportion of the curve (Fig. 3). This is in agreement with Dohleman and Long (2009) who demonstrated that increased yield of *Miscanthus* compared with maize was achieved through increased canopy duration and a positive association between seasonal light interception (ε_i in Equation 1). However, the influence of other factors such as temperature and water availability cannot be excluded.

Variance in the yield data increased with increasing canopy duration (Fig. 3B, D), indicating that at longer canopy durations the correlation between canopy duration and yield was reduced. The reduced correlation may be because at more optimal canopy durations, variation in canopy duration became less significant and variation in other traits associated with yield became more significant.

What is causing the increased variance in yield at high canopy durations?

Yield is a complex trait and is the product of phenological, morphological, and compositional traits. Canopy duration is a complex trait and accounted for only a proportion of the yield variation in the population studied, but was more highly correlated with yield than the constituent traits measured (Table 1). At higher canopy durations, other factors were responsible for yield variation and, consequently, to define an ideotype for improved yield it will be necessary to consider the effects of other traits. To explore trait combinations fully was beyond the scope of this study, but correlations between available trait data from other studies on the same 244 genotypes were examined. Correlations between yields and traits were analysed in two subgroups of *Miscanthus* genotypes with high canopy durations which were within a 10 d range and yield values across a 5-fold range. Both PCA

and trait correlations indicated that stem traits in particular were highly correlated with variation in yield in both years (Fig. 5). The two approaches demonstrate possible combinations of traits that in parallel with canopy duration achieve maximum yield. However, some traits are aliased and a single driving principle may be described by more than one trait. The PCA and correlation analysis demonstrate that there is some overlap between traits associated with variation in yield at high canopy duration and traits correlating with high yield. This confirms the findings of other studies that have shown plant height or canopy height to be a strong predictor of above-ground yield, but indicates the importance of other traits such as growth rate, tillering, and tuft diameter which correlate well in some studies and not others (Jezowski, 2008; Zub *et al.*, 2011). At longer canopy durations, the seasonal decline of temperature and solar radiation levels in temperate climates may create a ceiling for effective canopy duration. Other traits such as leaf architecture may become more significant for light capture at longer canopy durations. Of particular note in perennial crops is that nutrient remobilization to storage organs may confound yield correlations.

Is early canopy establishment or late canopy senescence more effective in promoting yield through increased canopy duration?

The use of canopy duration to improve yield could be achieved in bioenergy crops by extending the growing season so that crops either grew earlier in the year or senesced later. However, there are factors which need to be considered when adopting such strategies. For example, early canopy establishment may produce a canopy which is damaged by late frosts or is exposed to cold and high light, generating photoinhibition of photosynthesis. Canopy senescence is also necessary for nutrient mobilization, and delayed senescence, or stay-green, is associated with poor crop overwintering in *Miscanthus* especially in the first winter (Jorgensen, 1997).

The relative contribution of early versus late season traits to increased yield was tested by calculating coefficients of correlation (Tables 1, 3). Significant and high correlations with yield were seen for all data. In both years, the coefficients were negative for canopy establishment and positive for canopy senescence, indicating that early canopy establishment and late canopy senescence were associated with increased yield. Canopy establishment correlated slightly better with yield than did canopy senescence in both years. *Miscanthus* stay-green genotypes were among the highest yielding in a previous study of *Miscanthus* growing at different sites across Europe (Clifton-Brown *et al.*, 2001). However, the stay-green phenotype was latitude dependent and was correlated with high yield at lower latitudes, whereas at higher latitudes stay-green genotypes yielded poorly, possibly because recycling of nutrients via senescence was not completed before killing frosts occurred (Jorgensen, 1997).

In genotypes with the longest canopy duration, a subgroup of *M. sinensis* genotypes was identified in which canopy duration was strongly correlated with yield and with early canopy establishment. This suggests that although the *M. sinensis*

genotypic group was the lowest yielding on average and had the weakest correlations between canopy duration and yield (Table 3), within *M. sinensis* genotypes with the longest canopy durations, canopy duration driven by early canopy establishment correlated strongly with yield (Table 1).

The data presented in Tables 1 and 3 are in agreement with the observation in willow that early bud flush was more significant for improved yield than was delaying late season senescence (Ronnberg-Wastljung and Gullberg, 1999). However, these data contradict a study of 21 *Miscanthus* genotypes (Zub *et al.*, 2012) that showed that late establishment, short growth duration, and rapid growth were all correlated and were associated with high yield. Growth duration calculated by Zub *et al.* (2012) started earlier in the year than canopy duration calculated here. In estimating the impacts of growth season length in deciduous forest, White and Nemani (2003) demonstrated that the use of different phenological measurements to define growth season length could give different associations. Therefore, the discrepancy between the two *Miscanthus* studies may derive simply from the methodologies used or it may be of more fundamental interest. If we assume the two studies should generate roughly equivalent conclusions, one possible explanation for the discrepancy is the inclusion in Zub *et al.* (2012) of more days early in the season, a time when plants are more likely to be exposed to lower temperatures and incident radiation, or low temperature and high light leading to photoinhibition of photosynthesis. Such days are perhaps not useful in generating net biomass gains and therefore may have generated correlations in favour of later growth initiation.

How can the phenotyping of canopy establishment be optimized?

Three methods used for assessing canopy establishment were compared. No definitive value of canopy establishment is known; therefore, the different methods were assessed by generating canopy durations and testing for correlations with yield. The highest correlations with yield were achieved using light interception (Fig. 6A); this method provided a high level of quantification but was very laborious, could only be performed during a narrow window either side of the solar zenith, was weather dependent, and was better suited to plot trials rather than spaced plant trials. A method using digital image acquisition was cheap, quick, less restricted by weather and solar periodicity, and images could be stored for subsequent analysis. Image analysis provided a high level of quantification (Fig. 6C). The analysis method used the adjustable RGB threshold similar to those methods previously described for determining vegetation indices and phenology (Ahrends *et al.*, 2009). The disadvantage of using images to assess canopy establishment was the level of operator interaction in quality assuring the detection algorithms; this is likely to improve with developments which combine both colour analysis and object-based segmentation to define crop versus weed leaf areas more intelligently (Laliberte *et al.*, 2007). The third method, visual assessment of canopy establishment (Fig. 5B), was relatively quick and correlated well with yield.

The disadvantage of visual assessment is that the method cannot be subject to further quality assessment, except by repetition or correlation with other methods, and the level of quantification is low at a resolution of 10 percentile bands.

How can the phenotyping of canopy senescence be optimized?

Senescence in single leaves can be easily monitored using, for example, a hand-held SPAD meter to measure relative chlorophyll content (Papasavvas *et al.*, 2008). This approach may be appropriate if the majority of yield is derived from a single leaf, as it is in wheat where 30–50% of assimilates for grain filling come from the flag leaf (Sylvester-Bradley *et al.*, 1990). It is more challenging to assess senescence across numerous leaves and stems by such a method and therefore whole-plant image analysis has the potential to provide a quick and quantifiable method that is suitable for senescence analysis in bioenergy crops. In this study, capturing suitable images from mature plants in dense field trials was not achieved. The problems of capturing images of canopy senescence relate to depth of field and capturing defined but large plant volumes in mixed plots; therefore, visual assessment of whole-plant senescence remained the best phenotyping option. If suitable images were generated, approaches have already been described to distinguish green and senescent vegetation (Laliberte *et al.*, 2007).

To test how well senescence scores relate to other end of season assessments such as growth cessation, senescence scores were compared with two measures of seasonal growth cessation: maximum canopy height and maximum stem elongation (Fig. 7). The highest correlations with yield occurred when elongation measurements were used to define the end of the growth season, and the more accurate stem elongation measure generated the highest correlation coefficients. This is not surprising since stem traits had the highest correlations with yield (Fig. 5).

The potential for optimizing canopy duration in bioenergy crops

The development of *Miscanthus* as a bioenergy crop requires strategies for optimizing both crop yield and quality. *Miscanthus* is harvested annually and the entire above-ground biomass is removed, creating a growth scenario that does not easily map on to yield improvement strategies applied to, for example, grain crops or trees. To optimize the period of active growth, a number of different traits may be optimized, as illustrated in Fig. 8. Early season traits include a low basal temperature for early initiation of meristem development in combination with frost-resistant shoots and cold-tolerant photosynthesis. Late season traits include delayed flowering, delayed senescence, and rapid remobilization of nutrients to the rhizome. The impact of even a few days shift in either early or late season traits can be significant. The impact of delaying senescence in *Lolium temulentum* leaves by just 2 d was estimated to increase net carbon fixation by >10% (Thomas and Howarth, 2000). Leaf area is regulated to optimize the balance between photosynthetic carbon fixation and carbon
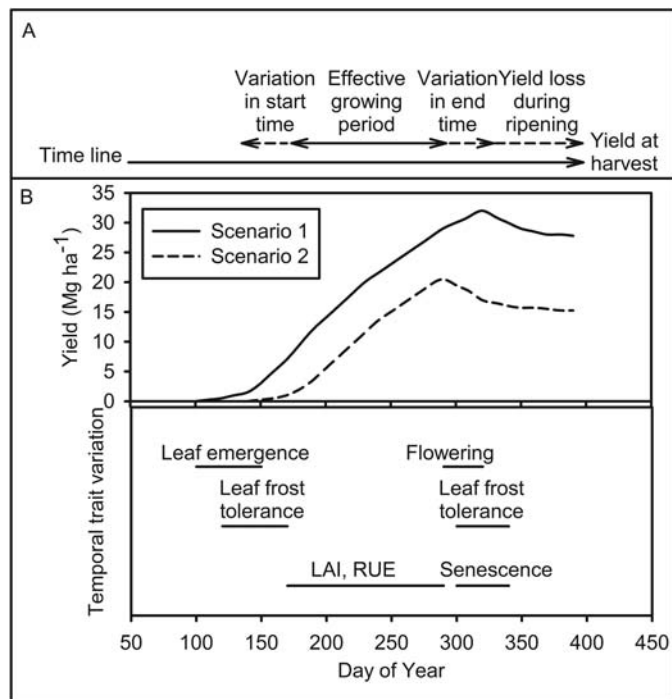
Fig. 8. A model of traits contributing to increased canopy duration and yield for a seasonally harvested perennial crop. (A) The effective growing period may be extended by earlier growth or delaying cessation of growth; however, after the growing season, a period of remobilization is required to improve crop quality and maximize growth in subsequent years. (B) In many environments, early emergence exposes shoots and leaves to low temperatures that may result in either frost kill or photoinhibition of photosynthesis; therefore, in addition to lower basal temperatures for the initiation of growth and leaf emergence, cold and frost tolerance will be required to ensure early emergence is beneficial to yield. Canopy architecture contributes to yield within the growing period but is not discussed herein. The effective growing period may be extended by delaying flowering and senescence; in some environments, frosts may damage frost-intolerant leaves, preventing active senescence processes. In higher latitudes, prolonged periods of delayed senescence result in poor yield, probably due to a combination of poor nutrient remobilization and net respiration in biologically active leaves in low light/temperature environments.

loss through respiration. It was hypothesized that defining canopy duration at the gross morphological scale, using traits that are associated with the regulation of leaf anabolism and catabolism, would identify positive trait to yield correlations that can be easily analysed at the whole-plant level.

To improve sustainable production of high yielding bioenergy crops such as *Miscanthus*, particularly for growing in temperate regions, it is proposed to rationalize the concept that every photon counts. The sun provides more energy to the Earth's surface in 1 h (4.3×10^{20} J) than human activity currently utilizes in 1 year (4.1×10^{20} J in 2001) (United Nations Development Program, 2003). The low efficiency of light energy fixation by photosynthesis in terrestrial plants is well documented (Zhu *et al.*, 2008). However, in extending the effective duration of

canopy development in dedicated bioenergy crops, it is ensured that more available energy is captured in an efficient manner. To improve annual biomass production in *Miscanthus* will require variation in traits associated with canopy duration from plants adapted to different latitudes and environments to optimize both early and late season development. It has been demonstrated here that this variation is available and that there is an association between canopy duration and yield in *Miscanthus*. The association between yield and canopy duration over two years has been demonstrated using combinations of individual canopy trait assessments. High canopy duration was found to be associated with considerable variance in yield in the population tested and this variance was associated with traits including leaf and stem morphology. The thresholds applied to traits allow the assessment to focus on appropriate canopy stages. The application of image analysis and other advanced phenomics techniques produced quantitative data that were relatively simple and consistent to acquire and that should transfer easily to quantitative analysis in breeding populations.

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